UNIVERSITI PUTRA MALAYSIA

CLONING OF GENES THAT ENCODE TRANSCRIPTION FACTORS THAT BIND TO THE FLORAL CHITINASE GENE (CHI2;1) PROMOTER OF TOMATO USING THE YEAST ONE-HYBRID SYSTEM

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CHAN PICK KUEN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy.

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Chairman : Associate Professor K. Harikrishna, Ph.D.

Faculty : Food Science and Biotechnology

Flowering plays an essential role in the plant's reproductive system and so has generated considerable research interest. Both the production and correct functioning of floral tissues are a prerequisite for the formation of seeds and fruits in all economically important agronomic and horticultural plants. Many flower-specific genes have been isolated and identified. Elucidation of the mechanisms that control flower-specific gene expression has led to the identification of their regulatory elements. These elements are useful for targeting other genes to floral organs at specific times during development. Targeting gene activity to specific floral tissues without affecting other portions of the flower is a very powerful tool for basic and applied studies. The ability to target specific gene expression is essential for discovery of function other related genes and for the selective manipulation of the flower. Some of the interesting characters for manipulation include reduced pollen allergenicity, increased flower longevity and flower numbers, and modified flower architecture. Previously, a promoter region of a tomato stylar endochitinase, Chi2;1 gene had been isolated (Harikrishna et al., 1996) and demonstrated to drive high level of expression in the pistil of transgenic plants. Hence, this study has been

tailored to isolate the transcription factors that bind to the Chi2;1 promoter and to identify specific binding regions within the promoter responsible for its binding.

The yeast one-hybrid system approach was used to isolate transcription factors that recognize elements within the Chi2;1 promoter. Out of 6.17×10^6 yeast transformants screened, thirteen putative positive clones were identified and isolated based on positive β-galactosidase assays. The DNA sequence of these clones was determined and compared to known DNA sequences in the GenBank database. Most of these clones did no have any significant homology with any known functional genes.

Expression studies were conducted on three clones, with two clones, LN2-1-1 and LN2-3-1 isolated from the –446 to –680 promoter region of Chi2;1 and one, 90-2-1 from the –211 to –445 region of the Chi2;1 promoter. LN2-1-1 and 90-2-1 are predominantly expressed in pistils at anthesis with lower expression in petals. Meanwhile, the expression of LN2-3-1 was detected in both vegetative and floral organs. The temporal and spatial expression patterns of LN2-1-1 and 90-2-1 were similar to Chi2;1 (Harikrishna et al., 1996). *In situ* hybridization of the LN2-1-1 and 90-2-1 clones revealed that the mRNA of both genes were localized to the transmitting tissue of the mature tomato pistil as with the mRNA of the Chi2;1 gene (Gasser et al., 1989). The binding ability of the proteins encoded by both of these genes to the respective DNA sequences has been shown through a mobility shift assay. Furthermore, the protein encoded by 90-2-1 has been shown to localize to the nucleus and bind specifically to a 20 base pair sequence within the Chi2;1 promoter. These results suggest that both LN2-1-1 and 90-2-1 might interact with the Chi2;1

promoter region and influence Chi2;1 gene expression to a certain extent. However, the functions of these genes in mediating style-specific expression is still to be confirmed by transgenic analysis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGKLONAN GEN-GEN YANG MENGEKODKAN FAKTOR-FAKTOR TRANSKRIPSI YANG MENGIKAT PADA PROMOTER GEN BUNGA ENDOKITINASE (CHI2;1) DARIPADA TOMATO MENGGUNAKAN SISTEM YIS SATU-HIBRID

Oleh

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Bunga yang memainkan peranan penting dalam sistem reproduksi tumbuhan telah menjana minat yang mendalam terhadap penyelidikan. Penghasilan dan pengawalan fungsi bunga yang betul adalah wajib untuk penghasilan biji benih dan buah bagi semua tumbuhan yang penting dalam agronomi dan hortikultur. Banyak gen bunga-spesifik telah disaring and dikenalpasti. Penerangan mekanisma-mekanisma yang mengawal ekspresi gen bunga-spesifik telah membawa kepada penemuan elemen-elemen pengawalan. Elemen-elemen ini adalah penting untuk penyasaran gen-gen lain kepada organ bunga pada masa yang spesifik semasa tumbesaran. Sasaran aktiviti gen kepada organ-organ bunga yang spesifik adalah satu kaedah yang penting untuk penyelidikan asas dan gunaan. Kebolehan untuk menyasar ekspresi gen yang spesifik adalah perlu bagi penemuan gen-gen yang mempunyai fungsi yang berkaitan dan manipulasi yang spesifik pada bunga. Antara ciri-ciri menarik untuk manipulasi termasuk pengurangan alergi terhadap debunga, penambahan jangka hayat bunga dan bilangan bunga dan pengubahsuaian arkitektur bunga. Sebelum ini, satu promoter gen endokitinase, Chi2;1 daripada pistil tomato telah

dipencilkan (Harikrishna et al., 1996) dan berupaya untuk mengarahkan ekspresi yang tinggi di tisu pemindahan bunga. Justru itu, kajian ini telah disesuaikan untuk pemencilan faktor-faktor transkripsi yang mengikat kepada promoter Chi2;1 dan mengenalpasti bahagian yang spesifik dalam promoter yang bertanggungjawab terhadap aktiviti-aktivitinya.

Sistem yis satu-hibrid (yeast one-hybrid system) digunakan untuk pemencilan factor-faktor transkripsi yang mengikat kepada promoter Chi2;1. Daripada jumlah transformasi yis sebanyak 6.17×10^6 yang disaring, tiga belas klon putatif positif telah dikenalpasti berdasarkan perubahan warna biru apabila diuji dengan kaedah asai 'β-galactosidase'. Jujukan klon-klon ini ditentukan dan perbandingan dengan bank data 'Genbank' telah dilakukan. Kebanyakan klon-klon ini tidak mempunyai persamaan dengan sebarang gen berfungsi.

Kajian ekspresi ke atas tiga klon dengan dua klon, LN2-1-1 dan LN2-3-1 yang dipencilkan daripada bahagian –446 ke –680 promoter Chi2;1 serta 90-2-1 daripada bahagian –211 ke –445 telah dilakukan. Klon LN2-1-1 dan 90-2-1 menunjukkan ekspresi yang tinggi pada tisu pistil yang matang dan ekspresi yang rendah pada petal bunga. Manakala ekspresi klon LN2-3-1 boleh dikesan pada tisu-tisu vegetatif dan bunga. Corak ekspresi klon LN2-1-1 dan 90-2-1 adalah serupa dengan Chi2;1 (Harikrishna et al., 1996). Hibridasi *in situ* klon LN2-1-1 dan 90-2-1 mengesahkan lokasi mRNA kedua-dua gen di tisu pemindahan bunga pada pistil tomato yang matang seperti mRNA Chi2;1 (Gasser et al., 1989). Keupayaan mengikat protein yang diekspresikan oleh kedua-dua gen ini ditunjukkan melalui asai 'mobility shift'. Selain daripada itu, protein yang diekspresikan oleh 90-2-1

adalah ditempatkan di nukleus dan mengikat spesifik kepada 20 jujukan DNA dalam promoter Chi2;1. Keputusan-keputusan yang diperolehi mencadangkan kedua-dua LN2-1-1 dan 90-2-1 berkemungkinan berinteraksi dengan promoter Chi2;1 serta mempengaruhi ekspresi Chi2;1 pada suatu tahap. Walaubagaimanapun, fungsi sebenar gen-gen ini di dalam ekspresi gen benangsari-spesifik perlu dikenalpasti melalui analisis transgenik.

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I certify that an Examination Committee on 13th August 2003 to conduct the final examination of Chan Pick Kuen on her Doctor of Philosophy thesis entitled "Cloning Of Genes That Encode Transcription Factors That Bind To The Floral Chitinase Gene (Chi2;1) Promoter Of Tomato Using The Yeast One-Hybrid System" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not previously or concurrently submitted for any other degree at UPM or other institutions.

(CHAN PICK KUEN)

Date: 9/11/2003

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LIST OF ABBREVIATIONS

Symbol	Description
α	alpha
β	beta
λ	lambda
%	percentage
^{O}C	degree Centigrade
g	gravity
AD	activation domain
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
BSA	bovine serum albumin
Ci	curie
C-terminal	carboxyl terminal
Cys	cysteine
cDNA	complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DIG	Digoxigenin
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease
dNTP	deoxynucleotides
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate

dTTP	thymidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
DMF	N,N-dimethylformamide
DMSO	dimethylsulphonyl oxide
DTT	dithiothreitol
EtBr	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis-(β-aminoethyle ether)
g	gram
GUS	β-glucuronidase gene
HCl	hydrochloric acid
His	histidine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr	hours
IPTG	isopropyl-β-D-thiogalactoside
LB	Luria-Bertani
Leu	leucine
k	kilo
kb	kilobase-pair
KCl	potassium chloride
kD	kilodalton
L	liter
Leu	leucine
LiAc	lithium acetate
LiCl	lithium chloride

M	molar
mA	milliampere
mg	milligrame
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MS	Murashige and Skoog medium
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MOPS	3-(N-morpholino) propane-sulphonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
NBT	nitro blue tetrazolium
ng	nanogram
N-terminal	amino terminal
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pfu	plague forming unit
pI	isoelectric point
PEG	polyethylene glycol

PMSF	phenylmethyl-sulfonyl fluoride
poly A^+ RNA	polyadenylated RNA
psi	pounds per square inch
PVP	polyvinylpyrrolidone
PVPP	polypolyvinylpyrrolidone
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
sec	second
SD	synthetic dropout
SDS	sodium dodecyl sulfate
Streptavidin-AP	Streptavidin-Alkine Phosphatase
TAE	tris acetate EDTA
TBE	tris borate EDTA
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
U	unit
µl	microliter
µg	microgram
µm	micrometer
Ura	uracil
UV	ultra violet

V	volt
X-Gal	5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl-glucuronide

CHAPTER 1

INTRODUCTION

One of the many challenges in molecular genetics is to understand the molecular basis of gene regulation, as it involves the complex interaction of several components. The ability to regulate the expression of genes is fundamental to most biological phenomena such as development, differentiation, cell growth and responses to environmental signals. Transcriptional regulation of gene expression relies on the recognition of a *cis*-acting element by a transcription factor of the corresponding gene. The correct modulation of gene expression results in normal growth and development of an organism. Therefore, there has been a growing interest in gene regulation especially with tissue-specific genes. In tissue-specific gene regulation, a sequence specific DNA binding protein recognizes a *cis*-acting element of the corresponding gene. This binding will facilitate other components of the transcriptional machinery to initiate mRNA synthesis (Weinzierl, 1999).

Flowers play an essential role in the life cycle of flowering plants. Flowering is the first introductory step to fruit formation and is a fundamental part of the plants reproductive system. The range of events involved in flower development make flowering an excellent model system for understanding plant development in general. Understanding the nature of the protein products of tissue-specific genes and regulatory factors that control their temporal and tissue-specific expression will provide important insights into the mechanisms of flower formation and function.

The isolation and identification of regulatory regions of a floral tissue-specific gene, in conjunction with plant transformation will allow the targeted expression of genes to floral organs at specific times in their development. Introduction of these chimeric genes (appropriate promoter and desired gene) into plants will lead to better specific production of the gene product in floral organs.

The promoter concerned with in this study regulates a tomato floral stylar endochitinase, Chi2;1 (Harikrishna et al., 1996). The endochitinase was found to be expressed along the transmitting tract of the tomato style. Although the function of Chi2;1 has not been established, it is hypothesized to be involved in either facilitating pollen tube growth in the transmitting tissue or as a protective compound against pathogen (fungal) attack.

The Chi2;1 promoter was demonstrated to drive high level expression in the pistils of transgenic plants. The SK2 promoter, a potato homologue of the tomato Chi2;1 promoter, has been characterized by Ficker et al., in 1997. They have found that the regulatory elements directing expression were located within a 230 bp fragment. Therefore, it is anticipated that the promoter sequences of these two endochitinase genes would have high homology. The regulatory elements of the Chi2;1 promoter that interact to confer tissue-specific expression could possibly be located around the same region as the SK2 promoter.

It is hoped that the transcription factors that bind to specific *cis* regulatory elements can be identified and attempts to regulate genes through chimeric promoters in plants may be feasible through the use of bioinformatics and molecular